Kidney International, Vol. 57 (2000), pp. 1764–1769

TECHNICAL NOTE

Analysis of nanoliter samples of electrolytes using a flow-through microfluorometer

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Analysis of nanoliter samples of electrolytes using a flow-through microfluorometer. Several techniques have been developed to study the transport properties of nanoliter samples of renal tubule segments, such as continuous flow colorimetry and continuous fluorometry. We have extended the capability of the NANOFLO™, a flow-through microfluorometer, designed for measurement of carbon dioxide, urea, ammonia, glucose, lactate, etc., to analyze sodium, calcium and chloride ions, using three commercially available fluorescent indicators for intracellular and extracellular measurements. The selection of fluorescent indicator for each electrolyte was dependent on the optimal match of the dissociation constant and the analyte concentration range of interest. Using Fluo-3 dye we achieved a detection limit for Ca^{2+} of 0.1 pmol and selectivity over Mg^{2+} of between 7:1 to 10:1. Using sodium green dye we achieved detection limit for Na^+ of 12 pmol and a selectivity over K^+ of 40:1. The detection limit for Cl^- using lucigenin dye was 10 pmol. This technique can be readily adapted for the measurement of other physiologically important ultralow volume.

Studies of the transport properties of renal tubule segments have been enhanced by the introduction of continuous-flow colorimetry [1] and continuous-flow fluorometry [2]. These techniques can be applied to ultramicrovolume samples of tubule fluid. Continuous-flow methodology has enabled the detection of various analytes in minute nanoliter samples. These analytes include urea [3], ammonia [4], lactate [5], raffinose [6], and carbon dioxide [7].

During 1995, we developed a novel flow-through microfluorometer (WPI P/N NANOFLO™), based on unique liquid waveguide capillary technology [8, 9]. The design principle was similar to that described by Vurek [1, 2]. A number of improvements have been incorporated into the design described in the current study. The silicon tubing used for liquid transport was changed to quartz capillary tubing, which is impermeable to CO_2 and nonreactive with the reagents and enzymes used. A refrigeration system was designed to stabilize the reagent for a longer operation time. The fluorescent cell was changed to a liquid core waveguide type [8, 9] to enhance the detection sensitivity.

Further development of the technique using commercially available reagents designed for colorimetric applications permitted the accurate measurement of carbon dioxide, ammonia, urea, glucose, and lactose in liquid samples as small as 1 nL. The detection principle in each case was based on the observed change in the fluorescence of the reagent that occurred as a result of redox reaction of either nicotinamide adenine dinucleotide (NAD) or nicotinamide adenine dinucleotide (reduced form; NADH) [10]. More recently, the NANOFLO™ has been used in a number of novel studies investigating the process of HCO_3^- reabsorption and the process of NH_4^+ secretion [11–13].

We report here on our most recent results on extending the capabilities of NANOFLO™ for analysis of various electrolytes, including sodium, calcium, and chloride ions. This has been achieved by using the various commercially available fluorescent indicators for intracellular and extracellular measurements, and adapting them for use with the highly sensitive flow-through technique offered by the NANOFLO™.

METHODS

Equipment

A diagram of NANOFLO™ illustrating the principle of operation is presented in Figure 1. The reagent is loaded into the reservoir and then flows through the injection port and the fluorescence cuvette. The fluorescent flow-through cuvette houses a patented liquid waveguide capillary cell (LWCC) [8, 9]. The flow rate is controlled by microprocessor syringe pump (SP210iw; World Precision Instruments, Inc., "WPI") operating in withdrawal mode. The nanoliter sample is injected into

Key words: transport, renal tubules, nanoliter sample, flow-through microfluorometer, intracellular dye, electrolyte, calcium, sodium, chloride, urea, ammonia, lactate, glucose, carbon dioxide.

Received for publication June 1, 1999
and in revised form September 29, 1999
Accepted for publication November 12, 1999

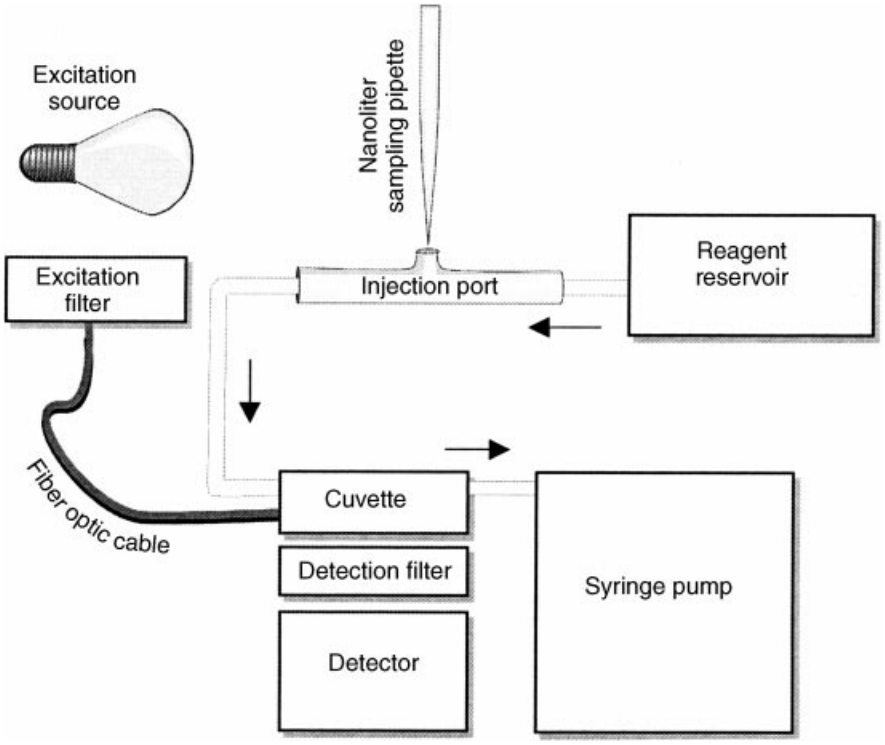

Fig. 1. Diagram of the NANOFLO™ flow-through microfluorometer, which is capable of measuring nanoliter samples. The excitation source is a stand-alone metal-halide lamp (Tri-Lite™; WPI, Inc.). The infusion/withdrawal syringe pump is SP210iw (WPI, Inc.). The principle of operation is explained in text.

the reagent flow through the injection port by using a horizontal stereo zoom microscope with a special holder, PZMH (WPI). The changes in the reagent fluorescence that occur are detected down the stream by the detector, which is a silicon photodiode (VISD; WPI).

The excitation source includes a metal halide excitation lamp (10,000-h bulb life-time; Tri-Lite™; WPI). The excitation and emission filters can be changed according to the reagents used. The analogue output of NANOFLO™ (0 to 5 V) was converted to digital format by an acquisition system Duo-18 (WPI).

Chemicals

Sodium green (tetramethylammonium salt; Molecular Probes, Inc., Eugene, OR, USA) was used for the detection of sodium ions. Fluo-3 (Molecular Probes, Inc.) was used for the detection of calcium ions. Lucigenin (Sigma, St. Louis, MO, USA) was used to detect chloride ion. Calcium chloride, magnesium chloride, sodium chloride, MOPS and HEPES buffers, tetramethylammonium hydroxide, and methanol were purchased from Sigma-Aldrich (St. Louis, MO, USA) and used as received. Phosphate buffer was from Micro Essential Laboratory (Brooklyn, NY, USA).

Preparation of reagents

Reagent for sodium ion. Ten mmol/L MOPS buffer was prepared and adjusted to pH 7.5 using tetramethylammonium hydroxide. Sodium green (tetramethylammonium salt; 64 μmol/L) in MOPS buffer was prepared for immediate use. NaCl-calibrating solutions were prepared in 10 mmol/L MOPS, pH 7.5, buffer.

Reagent for calcium ion. Fluo-3 (100 μmol/L) was prepared in 20 mmol/L, pH 7.3, phosphate buffer for immediate use. Calibration standards consisting of 0.5, 1.0, 1.5, and 2.0 mmol/L $CaCl_2$ solutions were prepared in 10 mmol/L MOPS buffer, 100 mmol/L KCl, and 1 mmol/L $MgCl_2$ solution. The pH was adjusted to 7.3 with NaOH.

Reagent for chloride ion. A 1 mmol/L solution of lucigenin was prepared in 10 mmol/L HEPES solution, pH 7.35. Calibration solutions of sodium chloride were prepared in 10 mmol/L HEPES solution, pH 7.35.

RESULTS

Figure 2 shows the results of an experiment for the detection of sodium ions in nanoliter samples using the dye sodium green. It had a maximum absorption at 509 nm and a maximum emission at 533 nm [14]. Figure 2A indicates the change in the fluorescence of the reagent with five successive 12 nL injections of 160, 80, 40, 20, and 10 mmol/L. The reagent sodium green increased its fluorescence upon binding with Na^+ [14]. After each injection, the background fluorescence recovered to the preinjection value. Depending on the configuration of the instrument and the flow rate of the reagent, the fluorescence peaks appeared between two- to five-minutes postinjection. Figure 2B shows a calibration curve

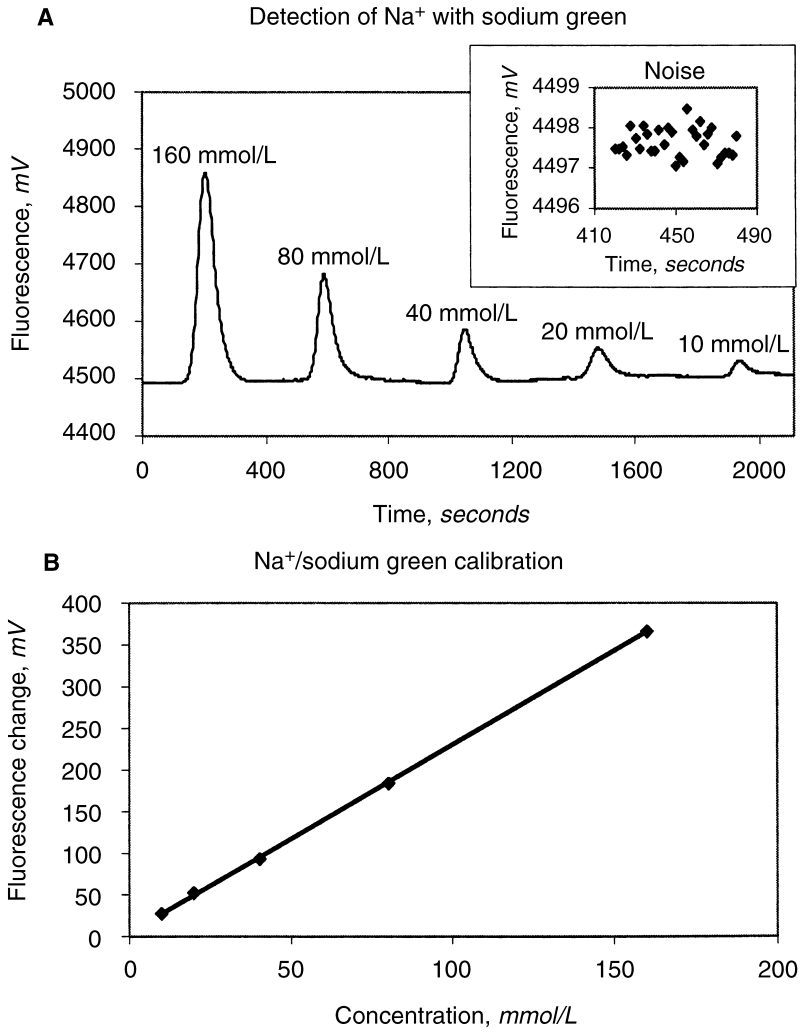

Fig. 2. Detection of Na^+ in nanoliter samples using NANOFLO™ and a reagent based on the sodium green dye (details are in the text). (*A*) Fluorescent trace showing the increase in the fluorescence of the dye caused by five subsequent 12 nanoliter injections of 160, 80, 40, 20, and 10 mmol/L NaCl. (*B*) Calibration curve based on the data in panel A. y = 2.2501x + 5.2333; $r = 0.9998$.

based on these results and illustrates the linearity that exists between the fluorescence and concentration. In this example, the gradient of the curve and thus sensitivity of the detection was 2.25 mV/mmol/L.

The insert in Figure 2A illustrates the background noise level between peaks. The standard deviation of the average value of the background is 0.3 mV. As the total variation in the signal was 0.6 mV and the slope of the calibration was 2.25 mV/mmol/L, the detection limit was estimated at 1 mmol/L (equivalent to 12 pmol in the sample volume used of 12 nL) at 3:1 to 4:1 signal to noise ratio.

Figure 3A illustrates the detection of Ca^{2+} with a reagent based on the dye Fluo-3. The maximum absorption was found at 503 nm and the maximum emission at 526 nm. Four different standard concentrations were injected (0.5, 1.0, 1.5, and 2.0 mmol/L). Fluorescence of Fluo-3 increased linearly with Ca^{2+} concentration. Figure 3A also illustrates the response of Fluo-3 to four different concentrations of Mg^+. Since Fluo-3 is also sensitive to Mg^+, the detection of Ca^{2+} was performed in the presence of 1 mmol/L concentration of Mg^+. Figure 3B shows the calibration curves for the results in Figure 3A. The sensitivity of detection of Ca^{2+} was 541 mV/mmol/L, and the detection limit was 8 μmol/L. This was equivalent to 0.1 pmol, based on 2 mV noise and a 2:1 signal to noise ratio.

Figure 4A illustrates the detection of various concentrations of Cl^- with the lucigenin dye. The fluorescence of lucigenin decreased in intensity with an increasing concentrations of Cl^-, producing the negative peaks observed in the fluorescence trace. The maximum absorption was at 368 nm, and the maximum emission was at 505 nm [15]. Figure 4B illustrates the Stern-Volmer plot generated using the data from Figure 4A. This graph demonstrates the linear relationship ($r = 1$) that existed when F_o/F was plotted as a function of ion concentration. F_o and F were background-corrected fluorescent levels before and after the addition of the sample. Based on Figure 4B, the detection limit was estimated at 0.8

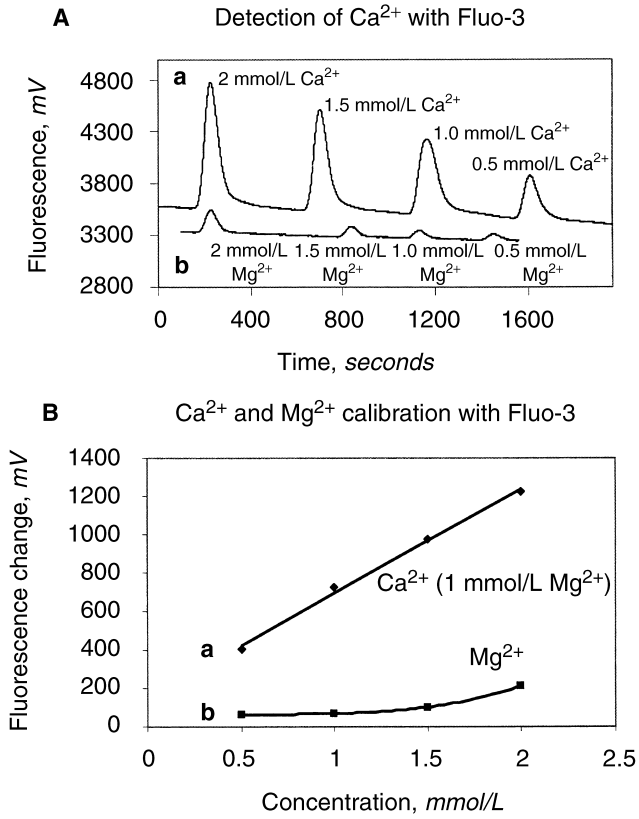

Fig. 3. Detection of Ca^{2+} in nanoliter samples using NANOFLO™ and a reagent based on the dye Fluo-3 (details are in the text). (*A*) Fluorescent trace showing the increase in the fluorescence of the dye caused by four subsequent 12 nL injections of (a) calibrating Ca^{2+} solutions (1 mmol/L Mg^{2+} present) and (b) calibrating Mg^{2+} solutions. (*B*) Calibration curves (a) and (b) are based on the data in (a) and (b) in panel A, respectively. Symbols are: (◆) Ca^{2+} (1 mmol/L Mg^{2+}); (■) Mg^{2+}. y = 540.8x + 155.5, r = 0.9964.

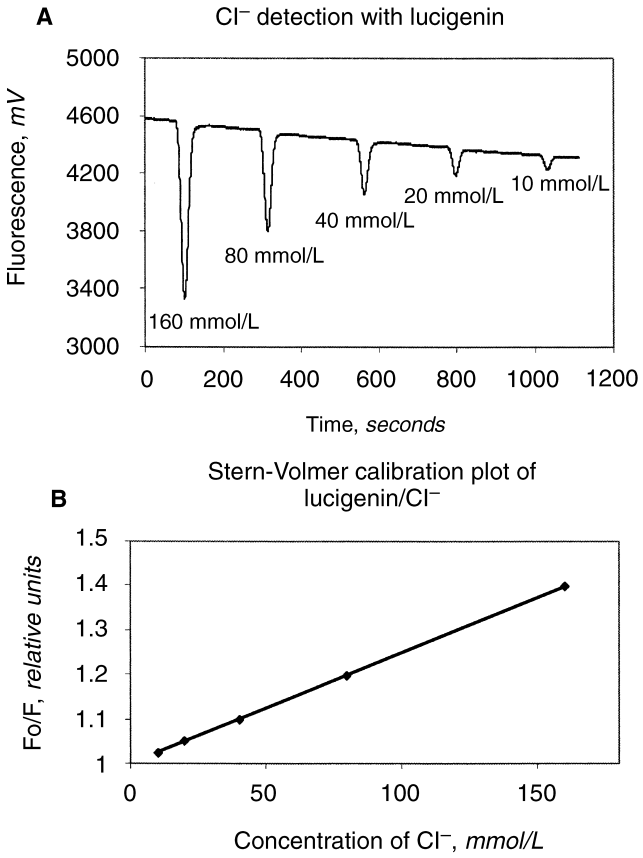

Fig. 4. Detection of Cl^- in nanoliter samples using NANOFLO™ and a reagent based on the lucigenin dye (details are in the text). (*A*) Fluorescent trace showing the decrease in the fluorescence of the dye caused by five subsequent 12 nL injections of calibrating Cl^- solutions. (*B*) Stern-Volmer calibration curve based on the data in (A). y = 0.0025x + 1.0008; r = 1.

mmol/L. This was equivalent to 10 pmol, based on 2 mV noise and a 2:1 signal to noise ratio.

DISCUSSION

The selection of a dye to be used as a reagent for the detection of an electrolyte depends to a great extent on the dissociation constant (K_d), which must be compatible with the concentration range of interest for the particular electrolyte. For example, most of the dyes for intracellular detection of Ca^{2+} are designed to respond to Ca^{2+} concentrations ranging from 50 nmol/L to 50 μmol/L. As the extracellular concentration of Ca^{2+} is in the 2.5 mmol/L range [2], it appears that Fluo-3, used for detection of Ca^{2+} in the present work (K_d = 390 nmol/L), is not suitable for the detection of extracellular Ca^{2+}. The reason we were able to measure extracellular Ca^{2+} by using Fluo-3 reagent in NanoFlo is that the injected sample was diluted, approximately 500 times, before it reached the detection cell, because of diffusion. Hence, the concentration of Ca^{2+} in the sample decreases down to 1 to 4 μmol/L, which is within the range of detection of Fluo-3.

Mg^{2+} has been found to interfere with the detection of Ca^{2+}. Extracellular concentrations of Mg^{2+} and Ca^{2+} are in the same range of 1.5 to 2.5 mmol/L [2]. We conducted experiments to establish the sensitivity of Fluo-3 to Mg^{2+}. These experiments indicated that Fluo-3 is between 7 and 10 times less sensitive to Mg^{2+} than it is to Ca^{2+} (Fig. 3). To compensate for this interference of Mg^{2+}, we recommend that the NANOFLO™ is calibrated for Ca^{2+} measurements in the presence of 1 mmol/L Mg^{2+} in the calibrating sample.

Sodium green exhibited an increase in fluorescence on binding with Na^+ and showed a 40-times greater selectivity for Na^+ than K^+. Assuming that in tubule fluid the concentration of Na^+ (140 mmol/L) is about 30 times larger than the concentration of K^+ (5 mmol/L) [2], we conclude that interference between Na^+ and K^+ would not be a problem. The typical extracellular concentration of Na^+ is 150 mmol/L. During the injection of a sample (before it reaches the detector), we have shown experi-

mentally that the sample is diluted approximately 500 times by diffusion. The resulting concentration is approximately 0.3 mmol/L. This is comparable to the K_d value of 6 mmol/L for sodium green. Hence, the measured concentration of Na^+ is within the sensitivity of sodium green.

The fluorescence of lucigenin is reduced by Cl^- (Fig. 4). The fluorescence was shown to be inversely proportional to the concentration of Cl^-.

The calibration curve (Stern-Volmer plot) illustrates a precise linear dependence, $r = 1$ (Fig. 4B). This enabled a very accurate determination of the unknown concentration. The slight decrease in the background fluorescence of the dye that is observed initially (Fig. 4A) is more than likely the result of photobleaching of the fresh dye reagent in the reservoir. This decrease can be substantially eliminated by covering the reservoir with aluminum foil to decrease the exposure of the reagent to light.

The Stern-Volmer quenching constant (K_{sv}) of lucigenin for Cl^- ions is 390 $(mol/L)^{-1}$ [14], while $1/K_{sv}$ is approximately 2.5 mmol/L. The latter represents the ion concentration that produces 50% fluorescent quenching. The typical extracellular concentration of Cl^- is 150 mmol/L. However, following injection, the sample is diluted, as explained earlier, to around 0.3 mmol/L. This concentration is comparable to the $1/K_{sv}$ value of 2.5 mmol/L. Hence, the measured concentration of Cl^- is within the sensitivity of lucigenin.

In experiments with biological samples, we recommend that the detection system be calibrated with solutions that have a chemical constitution that is very close to the chemical constitution of the measured sample. Since the fluorescent emission of many dyes depends on temperature and pH [14], it is very important to perform the calibration experiments under controlled conditions (physiological pH, room temperature or 36.6°C).

In the current study, we achieved a detection limit of 0.1 pmol for Ca^{2+} using Fluo-3. This is 10 times lower than the detection limit reported in previous studies based on colorimetry measurement with the reagent methylthymol blue [1]. The measured Ca in these experiments is ionized or free Ca. Fluo-3 only binds ionized Ca in solution. NANOFLO is primarily designed for measurement of various analytes in perfusate from renal tubules, which is an ultrafiltrate of blood plasma containing extracellular ions, glucose, and other small soluble molecules. Proteins such as immunoglobulins and albumin are absent from this ultrafiltrate. Albumin is the major complexing agent for Ca in blood plasma. Ca bound to albumin would not affect the measurement of free Ca^{2+}, since albumin is missing from the sample. This is not true if samples from blood plasma are used directly. In this case, the measured concentration value for free calcium is usually corrected based on the measured concentration of albumin, due to the dynamic equilibrium that exists between bound and free Ca.

The detection limit for Na^+ described here (12 pmol) is approximately five times lower than previously reported by using chromogenic macrocyclic ionophores [16].

The detection limit for Cl^- (10 pmol) is comparable to the detection limit reported by Star, Burg, and Knepper, using a reagent based on ferric thiocyanate [17], and Garcia, Plato, and Garvin [18], using a reagent based on the fluorescent Cl^- indicator, 6-methoxy-N-(3-sulfopropyl) quinolinium (SPQ).

Conclusion

We have described a convenient and highly accurate method for ultra low-level detection of Na^+, Ca^{2+}, and Cl^- in minute nanoliter samples of fluid using a novel flow-through microfluorometer (NANOFLO™) and a range of commercially available dyes (sodium green, Fluo-3, and lucigenin). The technique can be conveniently adapted to measure various other ultra low-volume analytes of physiological importance. There is currently no other economical and reliable method for measurements of this type using such minute volume samples.

It is envisaged that the method described here will stimulate greater research in this highly important field, and in doing so enhance the understanding of the physiological processes that occur within renal microtubules to the benefit of related medical studies.

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